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Influence of Primer Sequences and DNA Extraction Method on Detection of Non-O157 Shiga Toxin–Producing *Escherichia coli* in Ground Beef by Real-Time PCR Targeting the *eae, stx,* and Serogroup-Specific Genes†

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ABSTRACT

Non-O157 Shiga toxin–producing *Escherichia coli* (STEC) infections, particularly those caused by the ''big six'' or ''top six'' non-O157 serogroups (O26, O45, O103, O111, O121, and O145) can result in severe illness and complications. Because of their significant public health impact and the notable prevalence of STEC in cattle, methods for detection of the big six non-O157 STEC in ground beef have been established. Currently, the U.S. Department of Agriculture, Food Safety and Inspection Service detection methods for screening beef samples for non-O157 STEC target the stx_1, stx_2, and *eae* virulence genes, with the 16S rRNA gene as an internal control, in a real-time PCR multiplex assay. Further, the serogroup is determined by PCR targeting genes in the *E. coli* O-antigen gene clusters of the big six non-O157 serogroups. The method that we previously reported was improved so that additional *stx* variants, stx_{1d}, stx_{2e}, and stx_{2g}, are detected. Additionally, alignments of the primers targeting the *eae* gene were used to improve the detection assay so that *eae* subtypes that could potentially be of clinical significance would also be detected. Therefore, evaluation of alternative real-time PCR assay primers and probes for the *stx* and *eae* reactions was carried out in order to increase the *stx* and *eae* subtypes detected. Furthermore, a Tris-EDTA DNA extraction method was compared with a previously used procedure that was based on a commercially available reagent. The Tris-EDTA DNA extraction method significantly decreased the cycle threshold values for the *stx* assay ($P < 0.0001$) and *eae* assay ($P < 0.0001$), thereby increasing the ability to detect the targets. The use of different *stx* primers and probes increased the subtypes detected to include stx_{1d}, stx_{2e}, and stx_{2g}, and sequence data showed that modification of the *eae* primer should allow the known *eae* subtypes to be detected.

Non-O157 Shiga toxin–producing *Escherichia coli* (STEC) strains are increasingly reported as causing human infections in the United States. In some European countries, non-O157 STEC cause the majority of the STEC-associated illnesses *(15, 17)*. While *E. coli* O157:H7 appears to still cause many of the STEC-related outbreaks in the United States, it is estimated that non-O157 STEC strains cause more than twice the number of infections overall compared with *E. coli* O157:H7 *(9, 21)*. The number of illnesses caused by non-O157 serogroups has been compiled, and the Centers for Disease Control and Prevention (CDC) identified the top six most prevalent serogroups associated with illness (O26, O45, O103, O111, O121, and O145) *(9)*. Correspondingly, multiple Shiga toxin subtypes exist for stx_1 (stx_{1a}, stx_{1c}, stx_{1d}) and stx_2 (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) associated with STEC. A recent comprehensive study revealed that 24.3% of ground beef samples in the United States screened positive for *stx* by PCR *(8)*.

In addition, the intimin outer membrane protein, encoded by the *eae* gene, is another important STEC virulence factor and also has multiple variants and subtypes *(9, 17)*. The intimin gene (*eae*) was identified in 41.5% of the *stx* screen–positive beef samples in the United States and remains an important virulence factor when considering the development of detection assays *(8)*. Strains that carry the *eae* and stx_{2a} variant genes are more frequently associated with severe disease and hemolytic-uremic syndrome; however, STEC strains carrying other *stx* variants have also been associated with human illness *(25)*.

The sequences of the O-antigen gene clusters of the top six non-O157 STEC strains have been determined, and highly variable regions of genes, including the *wzx* gene (encodes for the O-antigen flippase) have been used to

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† Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

TABLE 1. *Accession numbers of* stx *variants analyzed*

stx type	Accession no.
1	Z36899, AM230663, Z36900, L04539, M23980, AY461172, AB083044, M16625, M19473, AB071619, Z360901, AJ312232, AJ314838, AB071623, AB048237, AJ314839, DQ449666, AY170851, AY986981
2	AM230664, EF441605, AY443048, AY443044, X07865, Z37725, EF441618, AF524944, AJ272135, AF461173, AY633471, EF441599, EF441609, AY443052, AY443054, AY443057, EF441613, Z50754, EF441619, AY633459, DQ143181, M59432, AY633473, AY443045, AY443043, L11079, AY633453, AY633467, AF291819, AY443049, AB015057, DQ235774, X61283, EU086525, L11078, EF441616, X65949, AJ567995, AF043627, AB048224, AB048225, AB048228, AJ567997, AB048238, AJ313015, AB048226, AB048223, AB048229, AF479829, FN182287, AF500187, AF500192, AF479828, AB071845, DQ235775, AF500190, DQ059012, AF500189, AF500191, AY443047, AJ313016, X81417, AY332411, AJ249351, U72191, X81415, X81416, X81418, AM904726, M29153, AB472687, AB232172, AJ010730, AY095209, AJ966782, AJ966783, AB048236, AB048227

design serogroup-specific real-time PCR assays *(12, 13)*. The use of the real-time PCR assays to identify the serogroup is a valuable molecular alternative to the antibody-based O-antigen detection methods.

Because of the serious health risks from non-O157 STEC infections, the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA FSIS) announced in November 2011 that the top six non-O157 STEC strains would be classified as adulterants in raw, nonintact beef products *(3)*. Testing for these pathogens began in June 2012. Therefore, sensitive and rapid screening assays will be in high demand in order to ensure the continued safety of our food supply. In 2010, 8,500 pounds of ground beef were recalled due to STEC O26 contamination and illnesses associated with beef contaminated with the pathogen *(2)*. The USDA FSIS *Microbiology Laboratory Guidebook* (MLG) chapter 5B.00 (released on 1 October 2010) described the procedure for detection and isolation of non-O157 STEC from enriched beef samples and used multiplex real-time PCR assays for detection of the stx_1, stx_2, and *eae* genes, with the 16S rRNA gene as an internal control. In addition, the procedure used PCR assays to determine the serogroup. However, the real-time PCR assay for detection of the stx_1, stx_2, and *eae* gene targets does not detect all known variants of these genes, leaving the potential for illness-causing STEC strains to go undetected. In this study, primer and probe sequences were designed to allow the detection of the additional stx_1 and stx_2 variants, as well as *eae* variants. Also in the study reported here, the genomic DNA preparation method was modified to increase the sensitivity of the real-time PCR assay. Finally, the real-time PCR assay was optimized for a higher throughput 96-well real-time PCR assay.

MATERIALS AND METHODS

Sequence analysis software. The accession numbers of sequences used in the alignments were obtained from GenBank and are listed in Tables 1 (*stx*) and 2 (*eae*). The sequences were chosen to represent each of the variants of these genes that are known. Alignments of the stx_1, stx_2, and *eae* genes were performed using the Clustal W alignment algorithm in the molecular evolutionary genetics analysis software version 5 (MEGA5) *(22)*. Figures 1 and 2, showing the partial sequence alignments, were created using Geneious software (Biomatters, Ltd., Auckland, New Zealand) *(11)*.

Strains used in this study. The *E. coli* strains used in this study are listed in Table 3. Some bacterial strains were kindly provided by the Michigan State University STEC Center, East Lansing. Bacterial strains used for exclusivity testing were from the American Type Culture Collection (Manassas, VA), from the USDA FSIS Eastern Laboratory strain collection (Athens, GA), and from the USDA Agricultural Research Service Eastern Regional Research Center Collection (Wyndmoor, PA).

Genomic DNA extractions from pure culture. Genomic DNA from the bacterial strains listed in Table 3 was prepared by

TABLE 2. *Accession numbers of* eae *(intimin) variants analyzed*

Accession no.
α (alpha) (M58154, AF022236, AB334565, AB334566, AB334567, FJ609829, FJ609806, FJ609805, FJ609798); α2 (AF530555, DQ523600); β (beta) (AF453441, AF200363, AJ277443); β2 (beta2) (AJ715407, DQ523605, FJ609816, FJ609807); β3 (beta3) (AJ876654, AJ876653); β4[a] (beta4) (FJ609802); γ (gamma) (Z11541, AJ875029, AF081185, AF081184, X60439, AF081183, AF081182, AF253561); γ2 (gamma2) (AF025311); δ[a] (delta) (AJ875027); ε (epsilon) (AF116899, DQ523606, DQ523614, AY186750, DQ523612, FJ609813); ε2 (epsilon2) (AF530554); ε3 (epsilon3) (AJ876650, AJ876649, FJ609827 (*E. albertii*)) ε4 (epsilon4) (AJ876651); ε8 (epsilon8) (FJ609810, FJ609811, FJ609814); ζ (zeta) (AF449417, AF449416, AJ298279, AJ271407); ζ2 (zeta2) (FM872420); ζ3 (zeta3) (FM872421, FM872422, FM872423); η (eta) (AJ308550, DQ523604); η2 (eta2) (AJ876652, AJ879900, AJ879899, AJ879898); θ (theta) (AF449419, AF449414, AF449415, AF449418, AF449420); θ2 (theta2) (FM872418); ι (iota) (AJ308551, DQ523601, DQ523602, FM872426, FJ609800); ι2 (iota2) (AF530553, AY696842 (*Shigella*)), κ[a] (kappa) (U66102, AJ308552, DQ523603, DQ523611); λ (lambda) (AF530557, AJ715409); λ2 (lambda2) (FJ609808); μ (mu) (AJ705049, DQ523607); ν (nu) (AJ705050, DQ523615 (*E. albertii*)), ξ (xi) (AJ705051, DQ523610); ο (omicron) (AJ876648); π (pi) (AJ705052); ρ (rho) (AJ748082, DQ523613, AJ748082, AJ748083, AJ748084); σ (sigma) (AJ781125); τ (tau) (AY696839 (*Shigella*), FM872416); υ (upsilon) (FM872417)

[a] β4, several κ's, and δ have zero amino acid differences.

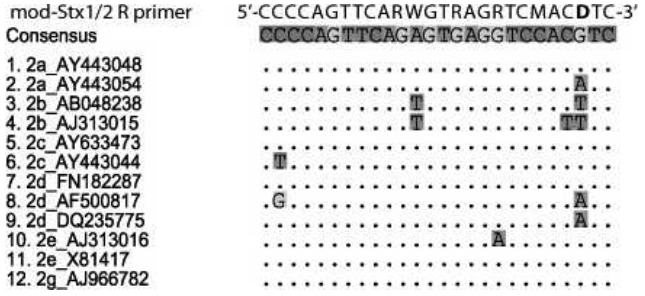

FIGURE 1. *Reverse primer modification for the* stx_2 *gene sequences. Sequences of the* stx_2 *variants were obtained from GenBank and were aligned using the Clustal W algorithm (MEGA5). Representative sequences for each variant with more than one sequence difference in this region are shown. The reverse complement strand is shown. Base 24 was modified to D (A, G, T) in order to have a higher level of homology to some of the variants.*

inoculating 100 μl of sterile water with a single colony from tryptic soy agar with sheep blood plates (Remel, Lenexa, KS), heating the bacterial suspensions at 99°C for 15 min to lyse the cells, and cooling before it was used in the PCR assays or stored at −20°C until use.

Ground beef enrichments used for evaluation of genomic DNA extraction methods. Uninoculated ground beef samples (325 g) were mixed with 975 ml of modified tryptic soy broth (Neogen Corp., Lansing, MI) (enzymatic digest of casein [17 g], acid digest of casein [10 g], sodium chloride [5 g], dipotassium phosphate [4 g], enzymatic digest of soybean meal [3 g], dextrose [2.5 g], and bile salts no. 3 [1.5 g], pH 7.3) and 20 mg/liter novobiocin in Whirl-Pak filter bags (Nasco, Ft. Atkinson, WI), mixed in a stomacher (Seward Laboratory Systems, Inc., Bohemia, NY) for 1 min, and incubated for 15 to 22 h at 42°C. To evaluate different methods for DNA extraction from the ground beef enrichments, the postincubation medium (enrichment) from uninoculated samples was inoculated with 10^5 CFU/ml of one of the six non-O157 STEC reference strains that had been grown on tryptic soy agar with sheep blood. The bacterial suspensions were made in 0.85% saline and then were serially diluted 10-fold in 0.85% saline before adding to the uninoculated beef enrichments. The bacterial dilutions were verified by plating onto Petrifilm aerobic count plates (3M, St. Paul, MN).

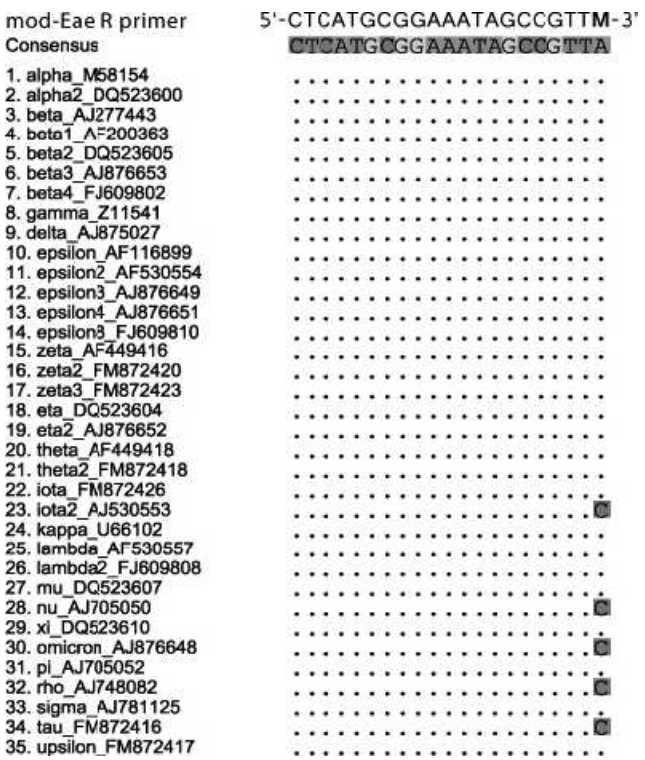

FIGURE 2. *Reverse primer modification for the* eae *gene sequences. Sequences of* eae *variants were obtained from GenBank and aligned using the Clustal W algorithm, showing the reverse complement strand. Based on the sequence information, base 21 was modified from the original primer format to M (A, C) so that all variants would have sufficient homology with this primer.*

Genomic DNA extractions from ground beef enrichments. For the Tris-EDTA (TE) extraction method, 1.4 ml of the inoculated enrichment was transferred to a microcentrifuge tube and centrifuged at 1,500 × *g* for 1 min to remove the larger debris. The supernatant was then transferred to a sterile microcentrifuge tube and centrifuged at 10,000 × *g* for 5 min, followed by resuspension of the cell pellet in 500 μl of 0.85% sterile saline to wash the pelleted cells. All centrifugation steps were performed at room temperature. Cells were centrifuged at 16,000 × *g* for 3 min, the supernatant was removed, and the pellet was resuspended in 90 μl of sterile TE buffer, pH 8 (Teknova, Hollister, CA) before heating at 95 to 100°C for 15 min. The tubes were cooled for 2 min and centrifuged at 16,000 × *g* for 4 min to pellet remaining debris before transferring the supernatant to a sterile microcentrifuge tube.

Preparation of genomic DNA using the PrepMan Ultra reagent (Life Technologies, Carlsbad, CA) was performed as previously described *(12),* with slight modifications. A 1.5-ml aliquot of inoculated enrichment was centrifuged at 2,000 × *g* for 1 min to pellet larger debris, and the supernatant was transferred to a clean tube and centrifuged at 16,000 × *g* for 3 min to pellet the cells. The cell pellet was resuspended in 200 μl of the PrepMan Ultra reagent. The solution was then heated for 10 min at 95 to 100°C, cooled, and centrifuged at 16,000 × *g* for 3 min before transferring the supernatant to a clean tube. Genomic DNA was used in the real-time PCR assays after preparation or was stored at −20°C until use. DNA analysis of the genomic DNA preparations obtained from artificially inoculated enrichment broths with either the TE- or PrepMan Ultra–prepared DNA was performed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Rockford, IL).

Real-time PCR assays for *stx, eae,* and O-antigen gene cluster targets. The primers and probes used in this study are listed in Tables 4 and 5 and were obtained from Integrated DNA Technology (Coralville, IA). All of the modifications made are described in the sections below. For the MLG chapter 5B.00 procedure for $stx_{1/2}$ and *eae* detection, the previously described *(12)* Stx1/2 primers and probes were used at the concentrations listed in Tables 4 and 5; Omnimix beads (Fisher Scientific, Pittsburgh, PA) and the SmartCycler (Cepheid, Sunnyvale, CA) with the cycling program consisting of 95°C for 120 s, and 45 cycles of 95°C for 10 s and 60°C for 45 s (optics on) were used to screen a collection of strains (Table 3). Primers and probes targeting the 16S rRNA gene *(12)* used as the internal control were included in the assays. Assays were run with 2.5 μl of genomic DNA, prepared from a single colony in pure culture, per 25-μl reaction. One replicate of each strain was tested by PCR.

The Applied Biosystems 7500 FAST (ABI 7500) real-time PCR instrument (Life Technologies) was used with the cycling parameters of 95°C for 10 min, followed by 45 cycles of 95°C for

TABLE 3. *Strains used in this study*

	Source[a]	Serogroup	Designation	Known *stx* and *eae*
1	ARS	O26:H11	HH8	stx_{1a}
2	ARS	O26:H11	SJ1	stx_1
3	ARS	O26:H11	SJ2	stx_1, stx_2
4	ARS	O45:H2	SJ7	stx_1
5	ARS	O45	RM2048	stx_2
6	ARS	O45:H2	SJ8	stx_1
7	ARS	O103:H2	GG7	stx_{1a}
8	ARS	O103:H25	SJ11	stx_1
9	ARS	O103:H11	SJ12	stx_1, stx_2
10	ARS	O111:NM	SJ13	stx_1, stx_2
11	ARS	O111:[H8]	EE5	stx_{1a}, stx_{2a}
12	ARS	O111	RM7527	stx_1
13	ARS	O121:H19	SJ18	stx_1, stx_2
14	ARS	O121	RM9982	stx_1
15	ARS	O145:NM	SJ23	stx_1, stx_2
16	ARS	O145:H28	E59	Neg[b]
17	ARS	O145:NM	BCL73	Neg[b]
18	ARS	O145:H^+	314-5	stx_1
19	ARS	O145:H18	07865	stx_2
20	ARS	O113:H4	FF6	stx_{1c}, stx_{2b}
21	ARS	O174:H8	AA1	stx_{1c}, stx_{2b}
22	ARS	O157:H7	JJ10	stx_{2c}
23	ARS	O177:[H25]	DD4	stx_{2c}, stx_{2d}
24	ARS	O91:H21	B2F1	$stx_{2d\ activatable}$
25	ARS	O138	TW05622	stx_{2e}
26	ARS	O120:H^-	K30-3C32	stx_{2e}
27	ARS	O120:H^-	K6-1C2	stx_{2e}
28	ARS	O63:H6	CB9764	stx_{2f}
29	ARS	O63:H6	CB10019	stx_{2f}
30	ARS	O128ac:[H2]	CC3	stx_{2f}
31	ARS	O55:H7	BB2	stx_{1a}
32	ARS	O41:H26	119	stx_{1d}
33	ARS	O145:NM	87.1009	Neg
34	CDC	O103:H11	NML 04-3973	stx_1
35	CDC	O121:NM	NML 03-4064	stx_2
36	CDC	O45:H2	05-6545	stx_1
37	MSU	O26:NM	DEC9F	Neg
38	MSU	O15:NM	RDEC-1	Neg
39	MSU	O111:H^-	ED-31	stx_1
40	MSU	O111:H^-	928/91	stx_1, stx_2
41	MSU	O111:H^-	TB226A	stx_1, stx_2
42	MSU	O70:H11	DEC10J	stx_1, stx_2
43	MSU	O104:H21	G5506	stx_2, eae^-
44	MSU	O91:H21	B2F1	stx_2, eae^-
45	MSU	O156:H21	M2113	stx_1,stx_2
46	MSU	O5:N	BCL17	stx_1
47	MSU	XO3:NM	90:1787	stx_2, eae^-
48	MSU	O45:H2	M105-14	stx_1
49	MSU	O121:H19	MDCH-4	stx_2, eae^-
50	MSU	O121	MT#18	stx_2
51	MSU	O103:H2	MT#80	stx_1
52	MSU	O111:H8	3215-99	stx_1, stx_2
53	MSU	O45:NM	DA-21	stx_1
54	MSU	O121:H19	MT#2	stx_2
55	MSU	O26:H11	97-3250	stx_1, stx_2
56	MSU	O145:[H28]	4865/96	stx_1
57	MSU	O26:N	TB352A	stx_1
58	MSU	O111:NM	3007-85	stx_1, stx_2
59	MSU	O145:NT	H16	stx_2
60	MSU	O157:H7	86-24	stx_2

TABLE 3. *Continued*

	Source[a]	Serogroup	Designation	Known *stx* and *eae*
61	MSU	O55:H7	DEC5D	Neg
62	MSU	O55:H7	3256-97	stx_2
63	MSU	O157:H7	EDL-933	stx_1, stx_2
64	MSU	O145:H16	DEC101	stx_1
65	FDA	O2	7v	stx_{2g}
66	CDC	O104:H4	2009EL-2071	stx_{2a}
67	CDC	O104:H4	2009EL-2050	stx_{2a}
68	CDC	O104:H4	2011C-3493	stx_{2a}
69	U of Washington	O26:H2	TB285	stx_1
70	CDC	O45:H2	96-3285	stx_1
71	U of Washington	O103:H2	Tarr	stx_1
72	CDC	O111:H$^-$	94-0961	stx_2
73	CDC	O121:H19	96-1585	stx_2
74	CDC	O145:H$^-$	94-0491	stx_1, stx_2

[a] ARS, Agricultural Research Service centers, Eastern Regional Research Center; CDC, Centers for Disease Control and Prevention; MSU, Michigan State University STEC collection; FDA, U.S. Food and Drug Administration; U of Washington, University of Washington.
[b] Neg, negative.

15 s and 59°C for 60 s (optics on). The thermal cycler was run in standard mode using Environmental Mastermix 2× (Life Technologies) as directed. Assays for screening the strain collection (Table 3) and inclusivity and exclusivity studies were run with 5 μl of genomic DNA, prepared from a single colony in pure culture, per 25-μl reaction as was done with the SmartCycler. One replicate of each strain was tested by PCR.

Assays using artificially inoculated enrichment broths were run on the ABI 7500, using 5 or 2.5 μl of extracted genomic DNA. To ensure that contaminants containing the PCR targets were not present in the matrix, an uninoculated negative control was also extracted and tested. Assays were set up using 96-well optical PCR plates (Life Technologies) for higher sample throughput capability.

Statistical analyses. Data were analyzed using SAS statistical software (SAS Institute Inc., Cary, NC), and values are expressed as the mean ± standard deviation (SD). Analysis of variance followed by Fisher's protected least significant difference post hoc

TABLE 4. *Real-time PCR primers used in this study*[a]

	Sequence 5′→3′	Reference	Concn in reaction (μM)
MLG-Stx1-150-F	GAC TGC AAA GAC GTA TGT AGA TTC G	*12*	0.25
MLG-Stx1-150-R	ATC TAT CCC TCT GAC ATC AAC TGC	*12*	0.25
MLG-Stx2-200-F	ATT AAC CAC ACC CCA CCG	*12*	0.25
MLG-Stx2-200-R	GTC ATG GAA ACC GTT GTC AC	*12*	0.25
Stx1/2-F	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	*19*	0.2, 1.25[b]
Stx1/2-R	CCC CAG TTC ARW GTR AGR TCM ACR TC	*19*	0.2
mod-Stx1/2-R	CCC CAG TTC ARW GTR AGR TCM AC**D** TC	*19* (modified)	1.25[b]
MLG-Eae-170-F	CTT TGA CGG TAG TTC ACT GGA C	*12*	0.2
MLG-Eae-170-R	CAA TGA AGA CGT TAT AGC CCA AC	*12*	0.2
Eae-F	CAT TGA TCA GGA TTT TTC TGG TGA TA	*18*	1
mod-Eae-R	CTC ATG CGG AAA TAG CCG TT**M**	*18* (modified)	1
16S RNA IC-F	CCT CTT GCC ATC GGA TGT G	*12*	0.2,[c] 0.16[b]
16S RNA IC-R	GGC TGG TCA TCC TCT CAG ACC	*12*	0.2,[c] 0.16[b]
Wzx158-O26-F	GTA TCG CTG AAA TTA GAA GCG C	*12*	0.25
Wzx158-O26-R	AGT TGA AAC ACC CGT AAT GGC	*12*	0.25
Wzx72-O45-F	CGT TGT GCA TGG TGG CAT	*12*	0.25
Wzx72-O45-R	TGG CCA AAC CAA CTA TGA ACT G	*12*	0.25
Wzx191-O103-F	TTG GAG CGT TAA CTG GAC CT	*12*	0.25
Wzx191-O103-R	ATA TTC GCT ATA TCT TCT TGC GGC	*12*	0.25
WbdI-O111-F	TGT TCC AGG TGG TAG GAT TCG	*12*	0.25
WbdI-O111-R	TCA CGA TGT TGA TCA TCT GGG	*12*	0.25
Wzx189-O121-F	AGG CGC TGT TTG GTC TCT TAG A	*12*	0.25
Wzx189-O121-R	GAA CCG AAA TGA TGG GTG CT	*12*	0.25
Wzx135-O145-F	AAA CTG GGA TTG GAC GTG G	*12*	0.25
Wzx135-O145-R	CCC AAA ACT TCT AGG CCC G	*12*	0.25

[a] Degenerate nucleotide codes as follows: Y (C,T), W (A,T), R (A,G), M (A,C), D (A,G,T), S (C,G).
[b] Modified *stx* and *eae* PCR concentrations per reaction.
[c] USDA FSIS MLG chapter 5B.00 primer concentration per reaction.

TABLE 5. *Real-time PCR probes used in this study*

	Sequence 5′→3′	Reference	Concn in reaction (μM)
MLG-Stx1-P	TexRd-TGA ATG TCA TTC GCT CTG CAA TAG GTA CTC-IAbRQ	*12*	0.1875
MLG-Stx2-P	TexRd-CAG TTA TTT TGC TGT GGA TAT ACG AGG GCT TG-IAbRQ	*12*	0.1875
Stx1-P	TexRd-CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA A-IAbRQ	*19*	0.125
Stx2-P	TexRd-TCG TCA GGC ACT GTC TGA AAC TGC TCC-IAbRQ	*19*	0.125
Stx1-P	FAM-CTG GAT GAT /ZEN/CTC AGT GGG CGT TCT TAT GTA A-IAbkFQ	*19* (modified dyes)	0.25
Stx2-P	FAM-TCG TCA GGC/ZEN/ ACT GTC TGA AAC TGC TCC-IAbkFQ	*19* (modified dyes)	0.25
MLG-eae188-P	FAM-CTG GCA TTT GGT CAG GTC GGG GCG-BHQ_1	*12*	0.125
Eae-P	MAXN-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC-IAbkFQ	*18* (modified dyes)	0.2
Wzx158-O26-P	FAM-TGG TTC GGT TGG ATT GTC CAT AAG AGG G-BHQ_1	*12*	0.15
Wzx72-O45-P	FAM-ATT TTT TGC TGC AAG TGG GCT GTC CA-BHQ_1	*12*	0.1875
Wzx191-O103-P	MAXN-AGG CTT ATC TGG CTG TTC TTA CTA CGG C-3IABkFQ	*12*	0.2
WbdI-O111-P	MAXN-TGA AGG CGA GGC AAC ACA TTA TAT AGT GC-3IABkFQ	*12*	0.2
Wzx189-O121-P	MAXN-CGC TAT CAT GGC GGG ACA ATG ACA GTG C-3IABkFQ	*12*	0.2
Wzx135-O145-P	FAM-TGC TAA TTG CAG CCC TTG CAC TAC GAG GC-BHQ_1	*12* (one base change)	0.2
16S rRNA IC-P	TYE665-GTG GGG TAA CGG CTC ACC TAG GCG AC-IAbRQ	*12* (modified dyes)	0.1
16S rRNA IC-P	CY5-GTG GGG TAA CGG CTC ACC TAG GCG AC-IAbRQ	*12*	0.125

test or paired *t* tests were used for determination of statistical significance ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Recent U.S. data show that the prevalence of non-O157 STEC strains belonging to different serogroups in beef products may be higher than previously estimated *(8),* and estimates of infections caused by non-O157 STEC strains indicate that they may account for a higher number of infections than are caused by *E. coli* O157:H7 *(21)*. In the current study a real-time PCR detection method was developed to not only enhance sensitivity and the ability to detect the different *stx* and *eae* subtypes but also to increase sample throughput in the laboratory.

Optimization of the ABI 7500 real-time PCR platform for non-O157 STEC detection. For testing large numbers of samples, a high throughput system for non-O157 STEC detection is useful for regulatory agencies and for the food industry. Therefore, the 96-well ABI 7500 FAST instrument was used to optimize the process for detection of $stx_{1/2}$ and *eae,* adapting the real-time PCR detection methods for this platform. Because it is important to have a screening assay that requires little manual adjustment by the analysts, the use of a PCR reaction mastermix that would allow optimal detection and minimal adjustment was explored. The previous MLG non-O157 STEC detection assays used Omnimix beads (mastermix) with probes labeled with Texas Red, 6-carboxyfluorescein (FAM), and Cy5 (a cyanine dye) (see Table 5). When this assay was run on the ABI 7500, there were baseline and threshold adjustments that were required for each run. The assays were very sensitive to small changes in reaction volumes, which is not ideal when multiple samples need to be analyzed simultaneously. Therefore, the addition of a passive reference dye was used to normalize the reactions, providing optimal baseline and threshold adjustment without any user changes. The Applied Biosystems Environmental Mastermix 2× used in the modified assay has a carboxy-X-rhodamine (ROX) passive reference dye to allow automatic adjustments for small pipetting and baseline differences. The Texas Red dye on the probes used in the MLG 5B.00 assays is detected in the same channel as the ROX passive reference dye in the Environmental Mastermix; therefore, new probes labeled with dyes more suitable for the channels on the ABI 7500 machine (MAX NHS [*N*-hydroxy-succinimide] ester and Fluorescein/Zen) were selected (Table 5). This ROX-containing mastermix also performed well when using a single cycling program for detection of the *stx, eae,* and O-antigen gene cluster

targets on the same plate, simplifying the process when multiple reaction types are run simultaneously. Thus, a single real-time PCR program also allows laboratories the flexibility for multiple *stx* and *eae* assays to be combined with the serogroup PCR assays for maximum efficiency. After enrichment and DNA extraction, the *stx* and *eae* multiplex assay requires approximately 127 min to complete, and the O-serogroup assays require approximately the same amount of time. Therefore, if one desired to run both assays simultaneously on the same samples, there would be a savings of 127 min. However, the cost would increase by performing both assays simultaneously, since the *stx* and *eae* assay was designed to be the initial screening assay, and only samples that are positive would be tested with the O-serogroup–specific assays.

Modifications to the real-time PCR primers and probes enhanced detection of *stx* and *eae* subtypes. Sequence alignment analysis of the stx_2 variants indicated that the previously used forward primer for stx_2 (MLG chapter 5B.00) *(1)* had a 3′ mismatch and 5′ mismatch in the probe of the stx_{2e} variant, indicating that this variant would not be detected (see Table 1 for accession numbers used for the comparisons) and the stx_{2f}, stx_{2g}, and stx_{1d} variants would likely also not be detected due to mismatches throughout the primer and probe sequences. Because of the increasing awareness of non-O157 STEC strains possessing different virulence gene profiles linked to human illness, the primers and probes for *stx* and *eae* described in MLG chapter 5B.00 were modified to allow detection of more variants of the *stx* and *eae* genes so that there would be a greater probability of detecting new strains containing the less common variants. The *stx* primer-probe combinations described by Perelle et al. *(19)* are an improvement over the MLG chapter 5B.00 *stx* primers and probes since they allow detection of the stx_{1d}, stx_{2e}, and stx_{2g} variants. A single base change at base 24 (Table 4) of the Stx1/2-R primer *(19)* was inserted to enhance binding to the stx_1 and stx_2 variant sequences (except for stx_{2f}). In Figure 1, the alignments of the stx_2 genes in the region of the Stx1/2-R primer with the different sequence representatives for each of the variants are shown. The 24th base in the primer was changed from R (A, G) to D (A, G, T) so that the four nucleotide variations at this base would have better primer homology when one of the alternate nucleotides is present. The modified primer was designated mod–Stx1/2-R.

The ability of the unmodified Stx1/2, mod-Stx1/2, and MLG-Stx primer-probe sets to experimentally detect *stx* variants in a collection of STEC strains is shown in Table 6. The unmodified Stx1/2 and mod-Stx1/2 primer-probe assays detected the isolates carrying stx_{2e}, stx_{2g}, and stx_{1d}, whereas the current MLG chapter 5B.00 *stx* assays did not detect any of the three variants, indicating that the sequence analysis correlated with the experimental data.

Modifications to the real-time assay added stx_{1d}, stx_{2e}, and stx_{2g} detection capabilities in addition to the *stx* variants already detected by the MLG 5B.00 assay (stx_1, stx_{1c}, stx_2, stx_{2c}, stx_{2d}), as well as several additional *eae* subtypes. Currently the only known variant that is not detected in the newly modified real-time PCR assay is stx_{2f}. The stx_{2f} variant shows more differences in sequence compared to the other stx_2 variants, and separate sets of primers and probes have been used in multiplex real-time PCR assays in order to detect all stx_2 variants *(5)*. Although STEC strains carrying stx_{2f} are rarely detected in humans, a few cases of illness associated with the presence of the stx_{2f} variant have been reported; however, several of those cases may be attributed to coexisting medical conditions (e.g., *Campylobacter* spp. infection) causing the symptoms *(16, 24)*. In the future, separate primers and probes targeting stx_{2f} can be added to the modified real-time PCR multiplex assays if strains carrying this subtype are determined to be a significant human health threat. Despite the inability of the modified assay to detect stx_{2f}, the addition of the three *stx* variants greatly increases the STEC detection capability compared to the MLG 5B.00 non-O157 STEC assay.

Sequence analysis of the available *eae* gene subtypes showed that several *eae* subtypes, including theta and gamma2, which have been identified in O111 STEC of both human and bovine origin *(7, 14, 20, 23)*, could be detected with changes made to the primers and probes. The alignments show that the previously used forward primer in the MLG 5B.00 assay has a 3′ mismatch in the sequences of theta and gamma2, which indicates that the assay would likely not detect these variants. Current USDA FSIS screening criteria require a positive PCR result for both *stx* and *eae* prior to performing the serogroup-specific PCR assays; therefore, modifications to the *eae* real-time PCR assay are needed to detect clinically significant O111 isolates, along with a positive *stx* PCR result. The *eae* primers and probe described by Nielsen and Andersen *(18)* were evaluated as an alternative for the MLG non-O157 STEC *eae* screening assay. However, sequence evaluations of multiple *eae* variants revealed that the *eae* reverse primer contained a 3′ mismatch for iota2, nu, rho, omicron, and tau *eae* subtypes (Fig. 2). Beutin et al. *(4)* previously demonstrated that the rho variant was not detected by these primers. Therefore, the *eae* reverse primer *(18)* was modified to include a degenerate nucleotide at the 3′ end so that the modified *eae* assays are predicted to allow for detection of all currently known subtypes of *eae* (Table 4 and Fig. 2), decreasing the chance of pathogenic non-O157 STEC being undetected in ground beef.

Sequence analyses indicate that some non–*E. coli* (*Shigella, Escherichia albertii*) may also contain *eae* subtypes (e.g., epsilon3, iota2, nu, and tau) (see Table 2 for a list of the accession numbers used) that may also be detected with the *eae* real-time PCR assay. The possibility exists that a single enrichment could contain both a *stx*-positive and *eae*-negative *E. coli,* as well as an *eae*-positive non–*E. coli* that would result in a positive PCR screen. However, altering the *eae* detection assay to exclude those *eae* subtypes would also risk not detecting *stx*-positive and *eae*-positive non-O157 STEC. With the current method, serogroup-specific PCR assays are performed after the *stx* and *eae* screening assay; and, in addition, immunomagnetic separation, colony isolation, and confirmatory PCR assays are used to isolate and identify target pathogens when these

TABLE 6. *Comparison of real-time PCR primers and probe in different detection methods for* stx *and* eae

				Ct				
	Source[a]	Serogroup	Known *stx* and *eae*	MLG *stx*[b]	*stx*[c]	mod. *stx*[d]	MLG *eae*[b]	mod. *eae*[e]
1	ARS	O26:H11	stx_{1a}	13.87	15.15	15.39	12.48	17.56
2	ARS	O26:H11	stx_1	14.02	15.34	14.67	12.39	17.13
3	ARS	O26:H11	stx_1, stx_2	13.34	14.05	13.63	13.03	17.50
4	ARS	O45:H2	stx_1	16.08	16.69	16.29	14.21	18.87
5	ARS	O45	stx_2	13.52	14.28	13.77	12.2	16.44
6	ARS	O45:H2	stx_1	15.97	15.83	15.81	14.06	18.33
7	ARS	O103:H2	stx_{1a}	13.34	13.35	13.98	11.95	16.24
8	ARS	O103:H25	stx_1	13.34	15.16	14.38	12.39	16.89
9	ARS	O103:H11	stx_1, stx_2	13.56	14.47	14.40	12.56	16.99
10	ARS	O111:NM	stx_1, stx_2	12.64	14.06	13.72	12.72	17.35
11	ARS	O111:[H8]	stx_{1a}, stx_{2a}	15.19	15.42	15.86	14.67	18.86
12	ARS	O111	stx_1	13.97	14.71	15.68	12.33	17.15
13	ARS	O121:H19	stx_1, stx_2	15.16	15.94	15.47	15.19	19.96
14	ARS	O121	stx_1	11.47	12.58	11.40	10.97	14.93
15	ARS	O145:NM	stx_1, stx_2	12.42	13.53	13.21	12.31	16.85
16	ARS	O145:H28	Neg[f]	Neg	Neg	Neg	12.06	15.97
17	ARS	O145:Nm	Neg	Neg	Neg	Neg	10.97	15.39
18	ARS	O145:H^+	stx_1	14.50	14.43	14.69	12.02	16.90
19	ARS	O145:H18	stx_2	13.58	14.8	13.83	11.22	16.00
20[g]	ARS	O113:H4	stx_{1c}, stx_{2b}	11.18	12.31	12.34	Neg	Neg
21[g]	ARS	O174:H8	stx_{1c}, stx_{2b}	12.78	12.43	13.67	Neg	Neg
22	ARS	O157:H7	stx_{2c}	12.05	12.36	12.93	11.24	15.61
23	ARS	O177:[H25]	stx_{2c}, stx_{2d}	11.66	12.65	11.86	11.49	15.80
24	ARS	O91:H21	$stx_{2d\ activatable}$	12.58	13.65	13.33	Neg	Neg
25	ARS	O138	stx_{2e}	Neg	14.15	14.70	Neg	Neg
26	ARS	O120:H^-	stx_{2e}	Neg	14.14	14.68	Neg	Neg
27	ARS	O120:H^-	stx_{2e}	Neg	14.84	15.61	Neg	Neg
28	ARS	O63:H6	stx_{2f}	Neg	Neg	Neg	11.42	16.03
29	ARS	O63:H6	stx_{2f}	Neg	Neg	Neg	13.22	17.71
30	ARS	O128ac:[H2]	stx_{2f}	Neg	Neg	Neg	10.82	15.19
31	ARS	O55:H7	stx_{1a}	11.95	13.2	13.26	11.18	15.90
32	ARS	O41:H26	stx_{1d}	Neg	15.94	17.88	Neg	Neg
33	ARS	O145:NM	Neg	Neg	Neg	Neg	12.39	17.19
34	CDC	O103:H11	stx_1	14.27	14.63	14.66	13.04	17.54
35	CDC	O121:NM	stx_2	12.68	14.01	13.11	11.63	16.58
36	CDC	O45:H2	stx_1	15.83	16.77	16.89	14.31	19.64
37	MSU	O26:NM	Neg	Neg	Neg	Neg	12.16	16.91
38	MSU	O15:NM	Neg	Neg	Neg	Neg	12.13	17.01
39	MSU	O111:H^-	stx_1	12.21	12.47	12.90	12.1	17.09
40	MSU	O111:H^-	stx_1, stx_2	12.92	14.03	13.44	13.02	17.85
41	MSU	O111:H^-	stx_1, stx_2	13.06	13.35	13.33	12.39	17.21
42	MSU	O70:H11	stx_1, stx_2	15.00	14.31	14.86	12.62	17.39
43	MSU	O104:H21	stx_2, eae^-	13.77	15.46	14.56	Neg	Neg
44	MSU	O91:H21	stx_2, eae^-	12.84	14.14	13.47	Neg	Neg
45	MSU	O156:H21	stx_1, stx_2	14.61	14.44	14.86	13.12	17.77
46	MSU	O5:N	stx_1	13.24	13.15	13.37	12.04	17.19
47	MSU	XO3:NM	stx_2, eae^-	13.50	14.39	14.12	Neg	Neg
48	MSU	O45:H2	stx_1	16.18	16.11	16.44	13.4	19.09
49	MSU	O121:H19	stx_2, eae^-	12.09	13.45	12.48	Neg	Neg
50	MSU	O121	stx_2	13.24	14.02	14.27	13.37	18.69
51	MSU	O103:H2	stx_1	15.36	15.11	15.31	13.3	18.12
52	MSU	O111:H8	stx_1, stx_2	13.45	15.4	14.82	14.53	19.89
53	MSU	O45:NM	stx_1	14.30	13.86	14.94	12.43	17.79
54	MSU	O121:H19	stx_2	15.82	16.56	16.03	15.39	20.72
55	MSU	O26:H11	stx_1, stx_2	13.72	14.18	14.48	13.37	18.68
56	MSU	O145:[H28]	stx_1	13.35	16.51	15.17	12.01	17.87
57	MSU	O26:N	stx_1	15.15	15.10	16.05	13.02	18.69
58	MSU	O111:NM	stx_1, stx_2	15.46	15.93	16.48	15.24	20.68

TABLE 6. *Continued*

	Source[a]	Serogroup	Known *stx* and *eae*	Ct				
				MLG *stx*[b]	*stx*[c]	mod. *stx*[d]	MLG *eae*[b]	mod. *eae*[e]
59	MSU	O145:NT	stx_2	15.39	15.88	16.25	14.41	20.06
60	MSU	O157:H7	stx_2	12.38	13.92	14.31	12.12	17.80
61	MSU	O55:H7	Neg	Neg	Neg	Neg	15.07	19.82
62	MSU	O55:H7	stx_2	14.18	16.57	16.09	13.28	18.56
63	MSU	O157:H7	stx_1, stx_2	14.57	12.78	15.63	14.33	19.76
64	MSU	O145:H16	stx_1	13.77	13.35	14.09	11.62	16.81
65	FDA	O2	stx_{2g}	Neg	19.63	20.05	Neg	Neg
66	CDC	O104:H4	stx_{2a}	15.41	19.50	16.89	Neg	Neg
67	CDC	O104:H4	stx_{2a}	14.77	17.63	17.19	Neg	Neg
68	CDC	O104:H4	stx_{2a}	16.58	17.76	17.85	Neg	Neg

[a] ARS, Agricultural Research Service; CDC, Centers for Disease Control and Prevention; MSU, Michigan State University STEC culture collection; FDA, U.S. Food and Drug Administration.
[b] Assay and conditions described in *(1)*.
[c] Described in *(19)*.
[d] Modified from *(19)*.
[e] Modified from *(18)*.
[f] Neg, negative.
[g] Assays containing only stx_1 or stx_2 probes were used to determine the specificity for each of the toxins. Both stx_1 and stx_2 were positive for these strains.

types of mixed samples occur. The original unmodified *eae* primers and probe *(18)* were not evaluated in this study because it was known, based on the alignments, that some variants would not be detected.

Available *eae* sequence analysis predicted positive results for all known *eae* subtypes; however, a comprehensive *eae* subtype isolate collection was not available for experimental testing. It is expected that the *eae* assay changes will greatly increase detection of the *eae* subtypes, although a more in-depth experimental analysis is needed to verify the sequence analysis.

Because of the larger number of variants expected to be detected by the primers and probes for both *stx (19)* and *eae (18)* compared with those described in MLG chapter 5B.00 in PCR reactions, modifications to the previously described *(18, 19)* primer sequences were made to include more variants. The changes to the primers are listed in Table 4; changes from the original published primers are shown in bold. The probe sequences *(18, 19)* remained the same, with only changes in the dyes and quenchers used on either the SmartCycler or the ABI 7500 thermal cyclers.

O-group real-time PCR assays transferred to the ABI 7500 platform. PCR assays targeting the O-antigen gene cluster *wzx* gene and *wbdI* for serogroup O111 that were used for this study for *E. coli* serogroup identification can be a valuable alternative to serotyping methods that rely on antibodies. In addition to the *stx* and *eae* virulence targets, the MLG chapter 5B.00 O-group gene PCR assays were also modified for use with the ABI 7500 instrument. The primer and probe sequences remained the same, with the exception of modifications to some of the dyes and quenchers used for the probe previously described *(12)* (Table 5). The O145 probe was modified slightly from what was previously reported *(12)*. An additional T was added at the 5′ end of the O145 probe to prevent unwanted quenching effects that may result from having a G at the 5′ location. As with the other assays tested with the ABI 7500 platform, the mastermix and real-time PCR programs were also changed (as described above) from the previously reported non-O157 STEC method for serogroup detection *(12)*.

Exclusivity testing of the modified *stx* and *eae* real-time PCR detection assays. In order to test that the modified primers and probes described in this study for *eae* and *stx* were exclusive in detecting STEC, a panel of seven non–*E. coli* strains were tested from pure cultures using the modified *stx* and *eae* detection assays. The tested strains included *Enterobacter aerogenes, Enterococcus faecalis, Salmonella* Paratyphi A, *Salmonella* Typhimurium, *Salmonella* Heidelberg, *Proteus mirabilis, Morganella morganii, Klebsiella pneumoniae, Enterobacter sakazakii, Enterobacter cloacae,* and *Citrobacter freundii*. All of the non–*E. coli* isolates were negative for *stx* and *eae,* indicating specificity to intended targets.

Effect of genomic DNA extraction methods for ground beef enrichments on real-time PCR detection. MLG chapter 5B.00 used a 1:3 (325 g/975 ml of medium) ratio for beef enrichments and a genomic DNA preparation method using the PrepMan Ultra reagent. In this study, the effectiveness of different genomic DNA extraction methods was evaluated using enrichments from 1:3 meat/medium ratios using the modified non-O157 STEC detection assays described in this study, in order to determine the most robust combination. Real-time PCR assays targeting the *stx, eae,* and O-antigen gene cluster genes with enrichments containing 10^5 CFU/ml of one of the six non-O157 strains were performed using the MLG chapter 5B.00 method using

TABLE 7. *Effect of two different DNA preparation methods from inoculated enrichment broths on detection of* stx *and* eae *genes using real-time PCR assays on the ABI 7500 and modified primers and probes*[a]

		Real-time PCR assay					
		stx$_1$/*stx*$_2$			*eae*		
	Strain no.	TE (5 μl)[b]	PrepMan (2.5 μl)	*P* value	TE (5 μl)[c]	PrepMan (2.5 μl)	*P* value
O26	69	28.11 ± 0.32	32.06 ± 0.27	<0.0001	26.31 ± 0.12	28.97 ± 0.09	<0.0001
O45	70	28.79 ± 0.27	31.70 ± 0.21	<0.0001	27.33 ± 0.14	29.49 ± 0.0.9	<0.0001
O103	71	28.94 ± 0.19	32.68 ± 0.18	<0.0001	27.44 ± 0.28	30.14 ± 0.18	<0.0001
O111	72	27.19 ± 0.26	30.21 ± 0.29	<0.0002	27.27 ± 0.23	29.63 ± 0.32	0.0005
O121	73	28.68 ± 0.22	31.24 ± 0.24	<0.0002	27.97 ± 0.25	30.12 ± 0.33	0.0008
O145	74	28.05 ± 0.07	30.42 ± 0.11	<0.0001	27.72 ± 0.16	29.48 ± 0.14	<0.0001

[a] Strain numbers from Table 3. Significance level set at $P < 0.05$. Values are the average Ct ± SD ($n = 3$). Ground beef enrichments contained 10^5 CFU/ml of the non-O157 STEC listed.
[b] Average Ct values are significantly different from the PrepMan Ultra–prepared genomic DNA for the *stx* assay ($n = 3$).
[c] Average Ct values are significantly different from the PrepMan Ultra–prepared genomic DNA for the *eae* assay ($n = 3$).

PrepMan Ultra for DNA extraction *(1)*. This genomic DNA preparation method was compared with a modified heat lysis method previously described *(10)*. Genomic DNA prepared using the PrepMan Ultra reagent (2.5 and 5 μl) in the 25-μl total volume PCR assays using the mod-Stx1/2 and *eae* primers and probes was evaluated to determine the optimal volume for the assays. The 5-μl volume of PrepMan Ultra–prepared genomic DNA in this experiment decreased the sensitivity of the *stx* assays (increased cycle threshold [Ct] values), often resulting in negative results. When the PrepMan-prepared genomic DNA volume was reduced to 2.5 μl, as used in MLG chapter 5B.00, the *stx* assays were all positive (Table 7). These results indicate that the larger volumes of the PrepMan Ultra–prepared DNA were not optimal with the real-time chemistry in the modified assays.

An alternate genomic DNA preparation method was investigated to determine if the DNA preparation method or larger volumes of template DNA had an effect on detection of the *stx* target. A modified TE buffer and heat lysis genomic DNA preparation method, which should not contain ingredients that inhibit the PCR, was chosen for the final resuspension buffer in the genomic DNA preparation to improve the sensitivity of the *stx* assays. This TE extraction method involved a low centrifugation step to remove larger debris from the ground beef enrichments, followed by centrifuging and washing the cells with 0.85% saline solution before a final resuspension in TE and heat lysis of the cells. When this method was used to prepare the genomic DNA, there was a significant difference in the Ct values of the *stx* assay ($P < 0.0001$), compared with the PrepMan-prepared DNA (2.5-μl volume) with the TE-prepared DNA resulting in lower Ct values (Table 7). The TE method performed significantly better than the PrepMan method in all of the *stx* ($P < 0.0001$) and *eae* ($P < 0.0001$) assays. The average decrease in Ct value between the TE (5 μl)– and PrepMan (2.5 μl)–prepared DNA was 3.09 Ct for *stx* and 2.30 Ct for the *eae* assays. The *eae* target was less sensitive to the changes in the DNA preparation method than *stx*; however, the difference in Ct values for both was large enough to warrant changing to the TE method for non-O157 STEC genomic DNA preparations.

Extractions of uninoculated ground beef enrichments seeded with 10^5 CFU/ml of an O26:H2 (TB285) STEC were

TABLE 8. *TE- versus PrepMan Ultra–prepared genomic DNA from enrichment medium inoculated with 10^5CFU/ml of O26:H2 (TB285) STEC*[a]

Sample	Concn (ng/μl)	Concn normalized for vol[b]	Purity (260/280 nm ratio)	*stx* PCR template vol		*eae* PCR template vol	
				2.5 μl	5 μl	2.5 μl	5 μl
PrepMan.1	254.2 ± 65.38	254.2 ± 65.38	1.55 ± 0.025	33.97 ± 0.27	40.53 ± 0.40	34.27 ± 0.067	35.93 ± 0.22
TE.1	282.8 ± 28.74	141.4 ± 14.37	1.93 ± 0.015	29.22 ± 0.23	28.16 ± 0.06	31.19 ± 0.24	30.28 ± 0.12
PrepMan.2	146.8 ± 6.65	146.8 ± 6.65	1.49 ± 0.021	33.58 ± 0.31	39.79 ± 0.31	34.57 ± 0.11	35.92 ± 0.098
TE.2	252.7 ± 2.05	126.4 ± 1.03	1.93 ± 0.005	28.37 ± 0.22	27.34 ± 0.15	30.49 ± 0.12	29.50 ± 0.22
PrepMan.3	214.8 ± 11.27	214.8 ± 11.27	1.53 ± 0.005	34.31 ± 0.36	39.94 ± 0.21	34.76 ± 0.21	35.65 ± 0.10
TE.3	206.0 ± 0.60	103.0 ± 0.30	1.90 ± 0.031	30.42 ± 0.28	29.65 ± 0.38	32.29 ± 0.25	31.57 ± 0.37

[a] PrepMan.1 to PrepMan.3 and TE.1 to TE.3 represent three different enrichments with three replicates each. Values are means ± SD ($n = 3$).
[b] Concentration was normalized to account for differences in template volumes because the TE-prepared DNA was in a final volume of 100 μl and the PrepMan Ultra–prepared DNA was in a final volume of 200 μl. Therefore the TE-prepared DNA was divided by two, in order for a yield comparison.

TABLE 9. *Effect of genomic DNA preparation method on the real-time PCR serogroup-specific assays for the O-antigen gene cluster genes using the ABI 7500*[a]

Serogroup	Strain no.	DNA preparation method (vol used)		*P* value
		TE (5 μl)[b]	PrepMan (2.5 μl)	
O26	69	27.90 ± 0.46	31.15 ± 0.32	0.0006
O45	70	28.13 ± 0.17	30.47 ± 0.14	<0.0001
O103	71	28.68 ± 0.32	33.72 ± 2.83	0.0377
O111	72	32.59 ± 0.84	37.11 ± 0.41	0.0011
O121	73	29.42 ± 0.22	30.96 ± 0.66	0.019
O145	74	29.17 ± 0.09	31.30 ± 0.19	<0.0001

[a] Strain numbers from Table 3. Significance level set at $P < 0.05$. Values are the average Ct ± SD ($n = 3$). Ground beef enrichments contained 10^5 CFU/ml of non-O157 STEC.
[b] Average Ct values with the TE method are significantly different from the PrepMan Ultra–prepared genomic DNA for that assay ($n = 3$).

performed using the MLG chapter 5B.00 method using PrepMan Ultra and were compared with the TE extraction, in terms of purity and nucleic acid concentration, to elucidate possibilities for the differences in the real-time PCR assay results. DNA preparations (three different enrichments, three replicates each) were measured using the Nanodrop spectrophotometer for purity and concentration, and the results are shown in Table 8. The PrepMan Ultra–prepared DNA showed a higher DNA concentration than the TE preparation and a purity between 1.49 and 1.55 (260/280 nm ratio). The DNA extracted using both preparation methods were then assessed for effectiveness in the modified *stx* and *eae* PCR assays using a volume of either 2.5 or 5 μl of template. The results are shown in Table 8. Despite the higher concentration of total DNA using the PrepMan Ultra reagent, the TE-prepared DNA gave lower Ct values for both *stx* and *eae,* indicating that factors other than total DNA concentration influenced the Ct values obtained using the MLG 5B.00 DNA PrepMan Ultra preparation method using the assay conditions described in this study. Using the PrepMan Ultra extracts, the Ct values for *stx* in the PCR assay were higher (ca. 6 Ct value difference) when using 5 μl of template DNA versus 2.5 μl, whereas this difference was not observed with TE extracts. For *eae,* Ct values were slightly lower (ca. 1 Ct value) when 2.5 μl of PrepMan Ultra–prepared template DNA was used versus a 5-μl volume. The better performance of the lower volume of PrepMan DNA extract in the PCR reactions correlates with previous studies that indicated that larger volumes of PrepMan can inhibit PCR reactions *(6)*.

To confirm that the subsequent PCR reactions for the O-group gene targets were also improved by the TE genomic DNA preparation method (5 μl) used in the modified *stx* and *eae* assays, the same TE method and PrepMan Ultra–prepared DNA (2.5 μl) from the *stx* and *eae* screen were used for O-group PCR assays (Table 9). The *wzx* (or *wbdI* for O111) gene target assays had an average decrease in Ct of 3.14 when prepared using the TE extraction method versus the previous PrepMan-prepared DNA method described in MLG chapter 5B.00, with the O103 assay showing the highest difference at 5.04 cycles and O121 showing the lowest difference at 1.54 cycles.

Here, we showed that the TE genomic DNA extraction procedure and real-time PCR assays were able to maintain a robust PCR reaction despite the potentially increased amount of inhibitors that could accompany the more concentrated ground beef enrichment samples. Alterations to the genomic DNA preparation method also allowed for more flexibility in template volumes, which can be used to increase assay sensitivity if needed. The use of standard, low-cost solutions (saline, TE buffer) also gives smaller laboratories the ability to perform the genomic DNA preparations without the need for commercially available, more costly extraction reagents.

Non-O157 STEC screening will likely increase owing to the recent classification of the big six STEC as adulterants in nonintact raw beef products *(3)*. The transfer of the newly modified *stx* and *eae* and the serogroup-specific real-time PCR assays to a high throughput platform will allow testing laboratories several advantages over the previous method. A larger number of samples can be processed, and the use of multichannel pipettes and/or robotic equipment for 96-well plate assays can decrease or eliminate hands-on time for analysts, increasing throughput and reproducibility and decreasing the possibility of human error. The universal real-time PCR cycling program allows multiple assays to be combined in a single run, reducing the time and cost that would be required if the different assays were carried out separately.

In summary, the real-time multiplex PCR assay described in this study has the ability to detect most of the *stx* and *eae* subtypes using a single set of primers for *stx,* a single set of primers for *eae,* and three probes (stx_1, stx_2, and *eae*), and primers and probe targeting the rRNA 16S internal control. This approach provides an ideal screening tool for STEC in beef. The assay chosen contains one less set of primers compared with the MLG 5B.00 non-O157 STEC assay because it uses a single common stx_1 and stx_2 primer set, therefore reducing overall assay cost without loss of sensitivity. Furthermore, the TE-based extraction method in addition to the use of the 96-well ABI 7500 platform provides a more sensitive and high-throughput system.

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